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Effect of Temperature and Salinity on *Vibrio* (*Beneckea*) *vulnificus* Occurrence in a Gulf Coast Environment

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Vibrio (*Beneckea*) *vulnificus* is a recently recognized halophilic organism that may cause serious human infections. Patients infected with *V. vulnificus* often have a history of exposure to the sea, suggesting that the organism may be a common inhabitant of marine environments. Twenty-one inshore sites around Galveston Island in the Gulf of Mexico were cultured for *V. vulnificus* over a 12-month period. The organism was recovered from all but one of the sites at some time during the study. It was frequently isolated during the summer and fall from environments of relatively low salinity (7 to 16 $^{0}/_{00}$). *V. vulnificus* was rarely isolated from any of the sites during the winter months, when water temperatures dropped below 20°C. In vitro growth characteristics of environmental isolates of *V. vulnificus* demonstrated salinity optima of 1.0 to 2.0% NaCl and a temperature optimum of 37°C. These growth characteristics may account for the seasonal and geographical variations in occurrence of the organism. Overall, the results of these studies indicate that *V. vulnificus* is commonly found in Gulf Coast environments and that the occurrence of the organism is favored by warm temperatures and relatively low salinity.

A halophilic bacterium associated with serious human infections was described in 1976 by Hollis et al. (2). This organism was similar to *Vibrio parahaemolyticus* except that it fermented lactose, and it was initially referred to as lactose-positive vibrio. The organism is now recognized as *Vibrio vulnificus*. *V. vulnificus* may cause rapidly progressive bloodstream infections, with a high mortality rate, or it may cause wound infections (1). Both types of infection are associated with consumption of seafood or exposure to marine environments (1). Four *V. vulnificus* infections have been encountered at the University of Texas Medical Branch, Galveston, over a 2-year period, and two of these infections were fatal (6, 8). All of the patients were exposed to marine environments around Galveston Island, Tex., and one patient acquired pneumonia and septicemia after inhalation of seawater (6). These findings prompted studies of the occurrence and distribution of *V. vulnificus* in sites around Galveston Island, and preliminary results indicated that the organism was a common inhabitant of these environments (6). The present studies indicate marked seasonal variation in the occurrence of *V. vulnificus* and suggest that growth of the organism is favored by relatively high temperatures and low salinity.

MATERIALS AND METHODS

Environmental sampling. Surface water samples were collected from 21 sites around Galveston Island (Fig. 1). The sites included a ferry landing (A), a beach on the ship channel (B and C), an open beach on the Gulf of Mexico (D, E, F, and G), a bayou (H), a yacht basin (I, J, K, L, M, and N), a ship harbor (P and T), a shrimp boat harbor (O and U), a seawall (Q and S) and a fishing pier (R). Salinity and temperature determinations were made at each site whenever samples were collected. Water samples were collected in a clean bucket, and specific gravity and temperature measurements were taken immediately. Specific gravity was measured with a hydrometer, and salinity values were calculated from the formula salinity ($^{0}/_{00}$) = (specific gravity − 1) × 1,323 (9). Temperature was measured with a calibrated thermometer. Samples for culturing were collected in sterile 250-ml polypropylene bottles by immersion of the bottles directly into the sampling site. Samples were transported directly to the laboratory for microbiological analysis. These water samples were cultured for *V. vulnificus* by direct plating and by filtration. For direct plating, thiosulfate-citrate-bile salts-sucrose (BBL Microbiology Systems, Cockeysville, Md.) agar plates were inoculated with sterile swabs. For filtration, 0.1-, 0.5-, 1.0-, and 5.0-ml volumes of each sample were diluted in a final volume of 20 ml of sterile 3% NaCl and filtered through 0.45-μm-pore-size membrane filters (Gelman Instrument Co., Ann Arbor, Mich.). The dilutions were made to facilitate an even distribution of colonies on the filters and to achieve a count of 30 to 100 colonies per filter. The inoculated filters were cultured on thiosulfate-citrate-bile salts-sucrose agar at 37°C for 18 to 24 h.

Identification of isolates. Green or blue-green colonies on thiosulfate-citrate-bile salts-sucrose agar were transferred to brain heart infusion agar plus 1% NaCl. After overnight incubation, colonies were tested for

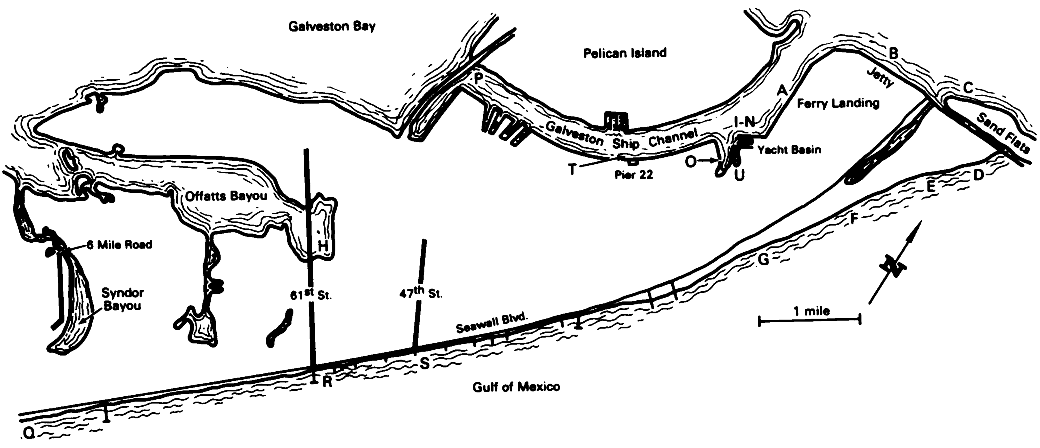

FIG. 1. Environmental sampling sites around Galveston Island. Twenty-one sampling sites are indicated by the letters A through U.

cytochrome oxidase reactions by a filter paper method with Kovács reagent. Oxidase-positive organisms were further screened for β-galactosidase and Voges-Proskauer reactions with PathoTec strips (General Diagnostics, Morris Plains, N.J.). Organisms that were β-galactosidase positive and Voges-Proskauer negative were inoculated into a biochemical test battery consisting of tests for lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, indole (tryptone broth tested with Kovács reagent), citrate utilization, fermentation of glucose and sucrose (phenol red broth base), and triple sugar iron agar. These media were supplemented with 1% NaCl to enhance growth of halophilic organisms. Salt tolerance was also determined with nutrient broth containing 0, 3, 6, or 8% NaCl. Environmental isolates giving reactions identical to those of previously reported human *V. vulnificus* isolates (6, 8) were recorded as *V. vulnificus*. These reactions included positive tests for citrate utilization, glucose fermentation, indole, β-galactosidase, oxidase, lysine, and ornithine decarboxylases, and growth in nutrient broth plus 3% NaCl and negative tests for arginine dihydrolase, sucrose fermentation, Voges-Proskauer reaction, and growth in nutrient broth plus 0 or 8% NaCl.

Determination of salinity and temperature optima Four environmental and two clinical isolates of *V. vulnificus* were tested to determine temperature and salinity optima for growth. Starting inocula containing 10^6 colony-forming units of the test organism per ml were prepared in brain heart infusion broth supplemented with 1.5% NaCl. Culture flasks (1,000 ml) containing 200 ml of inoculated broth were incubated without agitation at 42, 37, 35, 30, 25, and 13°C. Samples were collected at hourly intervals, and the optical density (at 540 nm) of each sample was determined. The optical density after 12 h of incubation (early stationary phase) was used for comparisons of growth at various temperatures. Salinity optima were determined in a similar manner with cultures in nutrient broth containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, or 6.0% NaCl; these cultures were incubated at 30°C on a shaker at 80 rpm. Salinity optima were also determined by an automated growth curve analysis with an MS-2 instrument (Abbott Diagnostics, Dallas, Tex.). These studies were conducted with nutrient broth containing various concentrations of NaCl.

RESULTS

Effect of temperature in situ. Environmental samples were collected at various times of the year and analyzed for physical characteristics and the presence of *V. vulnificus* to assess seasonal effects on the occurrence of the organism. Water temperatures varied widely over the year, ranging from a low of 12.5°C in December to a high of 31°C in August (Fig. 2). *V. vulnificus* was not recovered from samples collected in December, and it was rarely isolated in

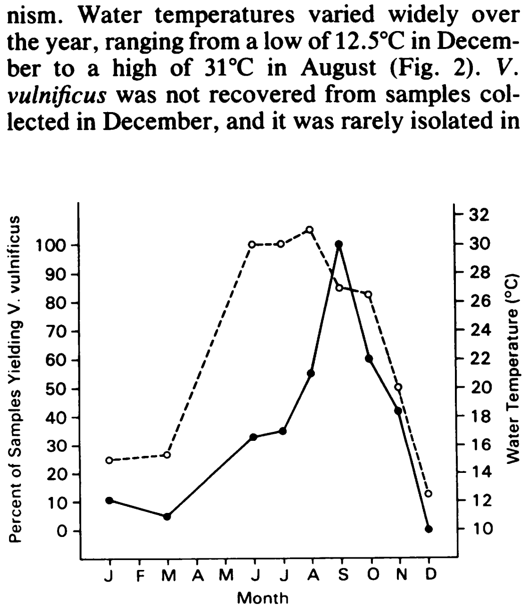

FIG. 2. Seasonal variation in the occurrence of *V. vulnificus*. Twenty-one environmental sites were sampled at monthly intervals. Symbols: ●, *V. vulnificus*; ○, water temperature.

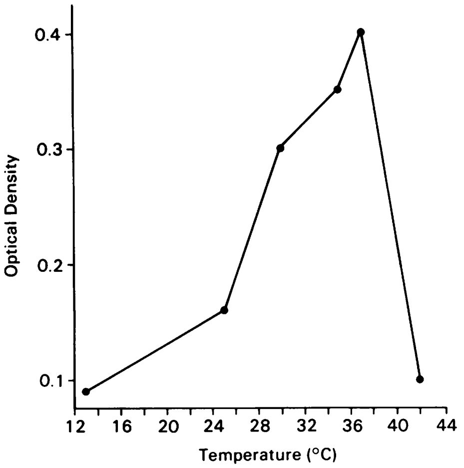

FIG. 3. Optimal temperature of *V. vulnificus* in vitro. Cultures were incubated at various temperatures for 12 h, and the optical density (at 540 nm) was determined. The results of a single representative experiment are presented.

January and March, when water temperatures were below 20°C. Recovery of the organism increased gradually through the summer months, when water temperatures averaged 30 to 31°C, and the peak recovery of *V. vulnificus* was noted in September, when water temperatures had been above 25°C for 4 months. Isolations of *V. vulnificus* rapidly decreased as water temperatures decreased in the fall.

Effect of temperature in vitro. The apparent seasonal variation in the occurrence of *V. vulnificus* suggests that growth of the organism may be favored by relatively high temperatures. To further investigate this possibility, I analyzed the growth of *V. vulnificus* isolates at various temperatures (Fig. 3). Maximum growth was obtained at 37°C, but the isolates also grew well at 30 and 35°C. Much slower growth was observed at 25°C, and no growth occurred at 13°C over the 12-h period of observation. However, after 72 h of incubation, slight growth was noted at 13°C. The isolates were not able to grow at 42°C. No differences were observed in the effects of incubation temperature on environmental versus clinical isolates of *V. vulnificus*.

TABLE 1. Sites frequently positive for *V. vulnificus*

Site	Description	Salinity (‰)	% Positive
A	Ferry landing	11.4	67
B	Ship channel	12.5	100
C	Beach (bay)	16.4	50
H	Bayou	8.6	50
J	Yacht basin	7.4	71
Avg		11.3	68

TABLE 2. Sites frequently negative for *V. vulnificus*

Site	Description	Salinity (‰)	% Positive
D	Beach (open gulf)	18.5	17
E	Beach (open gulf)	19.2	17
F	Beach (open gulf)	19.4	17
G	Beach (open gulf)	19.6	14
S	Beach (open gulf)	18.0	0
Avg		18.9	13

Effect of salinity in situ. To determine the effect of salinity on the occurrence of *V. vulnificus* in marine environments, I studied the Galveston Island sites intensively during the summer months, when the organism was likely to be present. The sites had average salinity values ranging from 6.5 to 19.6 ‰ during this period. The low salinity values were due to runoff of rainwater into areas protected from the open Gulf of Mexico. Five sites that frequently yielded *V. vulnificus* were identified (Table 1). These sites were all of relatively low salinity, and at least 50% of the samples collected from these sites were positive for *V. vulnificus*. Five other sites that seldom yielded the organism were identified (Table 2). These sites had an average salinity of 18.9 ‰, and less than 20% of the samples collected from them were positive. Overall, 47 ± 6% (mean ± standard error of the mean) of samples from sites having salinity values of <16 ‰ yielded *V. vulnificus*, and 22 ± 5% of samples from sites having salinity values of >16 ‰ yielded the organism (P = 0.02, Student's t test).

Effect of salinity in vitro. The results of the environmental studies suggest that growth of *V. vulnificus* is favored by relatively low salinity. To further investigate this possibility, I grew isolates of *V. vulnificus* in media containing 0.5 to 6% NaCl. Growth, as indicated by optical density after 12 h of incubation, was optimal in media containing 1.0 to 2.0% NaCl, and no growth occurred in media containing <0.1% NaCl or >5.0% NaCl. Similar experiments were conducted by an automated growth curve analysis. The optimal growth rate was found in media containing 0.5 to 2.0% NaCl (Fig. 4). Growth was retarded in media containing 4.0 to 6.5% NaCl, and no growth was noted in the presence of 8.5 or 10.5% NaCl. No differences were observed in the effects of salinity on clinical versus environmental isolates of *V. vulnificus*.

DISCUSSION

It was found in a previously published study that *V. vulnificus* is a common organism in Gulf

Coast environments around Galveston Island (6). The present findings confirm the earlier observations and also indicate that the occurrence of the organism is influenced by temperature and salinity. Marked seasonal variation in the occurrence of *V. vulnificus* was noted; the organism was seldom recovered from cold water but was often recovered when water temperatures were above 25°C. The incidence of recovery of the organism increased steadily during warm-water periods, and the peak incidence was noted in September, after water temperatures had been above 25°C for several months. These findings suggested that *V. vulnificus* grew best at elevated temperatures in situ, and this suggestion was confirmed by in vitro studies demonstrating an optimal growth temperature of 37°C.

The present results also indicated that salinity had an influence on the occurrence of *V. vulnificus*. Sites of low salinity often yielded the organism, but sites of relatively high salinity were seldom positive. With a salinity cutoff point of 16 ‰, a statistically significant difference in the rate of recovery of the organism was observed between sites of <16 ‰ and sites of >16 ‰ salinity. These findings suggested that the growth of *V. vulnificus* was favored by conditions of low salinity in situ, and in vitro studies indicated that the optimal salinity of the organism was <2% NaCl.

Similar effects of temperature and salinity have been found for other estuarine bacteria that have human pathogenic potential. As with *V. vulnificus*, the occurrence of *V. parahaemolyticus* was influenced by seasonal variations (4) and salinity (10). The incidence of isolation of *V. cholerae* from Chesapeake Bay was unaffected by seasonal variations in water temperature but was strongly influenced by salinity (5). My results suggest that *V. vulnificus* may closely resemble *V. parahaemolyticus* in its response to environmental temperature and salinity.

The relatively high temperature and low salinity optima of *V. vulnificus* suggest that this organism is distinct from other coastal marine bacteria, such as *Leucothrix mucor*, which typically have lower temperature and higher salinity optima, even when isolated from warm-water environments (7). *V. vulnificus* appears to be adapted to low-salinity estuarine environments and to growth at relatively high temperatures. The unusually high temperature optima of *V. vulnificus* and other human pathogenic marine bacteria may be important in the ability of these organisms to produce human infections. However, these characteristics also suggest that many marine environments of normally high salinity and low temperature may be hostile to *V. vulnificus*. This organism may grow in select, localized environments where optimal conditions

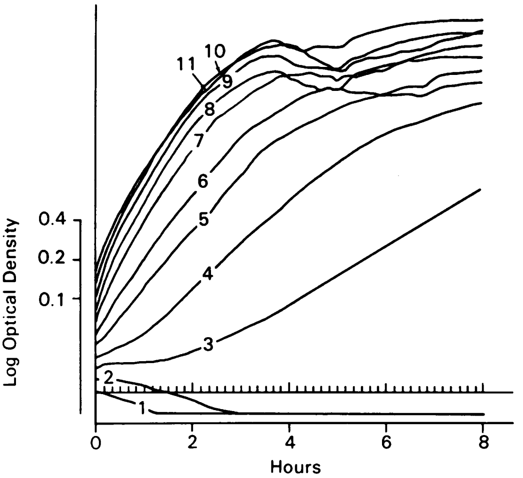

FIG. 4. Optimal salinity of *V. vulnificus* in vitro. Isolates were incubated in media of various salinity, and growth was monitored by an automated system. Individual growth curves were plotted on an expanded scale to allow visualization of each curve. The NaCl content of growth media used to generate each curve was as follows: 1, 10.5%; 2, 8.5%; 3, 6.5%; 4, 5.5%; 5, 4.5%; 6, 4.0%; 7, 2.5%; 8, 2.0%; 9, 1.5%; 10, 1.0%; and 11, 0.5%. The results of a single representative experiment are presented.

exist and then be disseminated to other environments by tidal flow or freshwater runoff. Studies are currently under way to test this hypothesis. A brackish water lake on Galveston Island connected to Galveston Bay by a tidal creek is being studied. Preliminary results have demonstrated a gradient of *V. vulnificus* with high colony counts in the lake and decreasing counts in the tidal creek and open bay (M. T. Kelly, manuscript in preparation).

The salinity and temperature effects on the occurrence of *V. vulnificus* are also interesting in relation to the incidence of human infections due to this organism. Eighty-five percent of *V. vulnificus* infections occur in the warm months of the year (1), and all of the infections detected at the University of Texas Medical Branch, Galveston, have occurred between May and October. These observations are closely correlated with the peak incidence of *V. vulnificus* in the environment. The mechanism of the seasonal variation in the occurrence of this organism in patients and the environment may be similar to that described for *V. parahaemolyticus*. This organism overwinters in bottom sediments and enters the water column again when warm temperatures return (3). The possibility that a similar mechanism exists for *V. vulnificus* is currently under investigation by analysis of sediment cultures collected from Galveston Island environments.

In conclusion, *V. vulnificus* is a human pathogen capable of producing highly lethal bloodstream infections and destructive wound infections (1). All evidence suggests that infections due to this organism are acquired from seawater or seafood (1, 6). My findings suggest that Gulf Coast environments may be a source of *V. vulnificus* and that the occurrence of the organism is especially favored by warm-water environments of low salinity. Such environments may pose a significant hazard for the acquisition of *V. vulnificus* infections.

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